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Short communication

Examination of growth inhibitory properties of synthetic chalcones for which antibacterial activity was predicted

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Abstract

A large series of chalcones were synthesized and studied against *Staphylococcus aureus* and *Escherichia coli*. Chalcones were either unsubstituted in ring A or possessed 4′-chloro or 3′,4′,5′-trimethoxy groups. Their other ring B was variously substituted. It was found that the anti-staphylococcal activity of chalcones was related to the energy difference between the two highest occupied molecular orbitals (HOMO and HOMO-1). Presence of hydroxyl group in ring B was not a determinant factor for the anti-staphylococcal activity, but the lipophilicity of ring A of the hydroxyl chalcones was of importance.

Keywords: Chalcones; Antibacterial activity; *Staphylococcus aureus*; *Escherichia coli*; SAR

1. Introduction

Chalcones are open-chain flavonoids with common skeleton of 1,3-diaryl-2-propen-1-one. Their wide-range biological properties, including antimicrobial activities, are largely attributed to the α,β-unsaturated ketone moiety. Introduction of various substituents into the two aryl rings is also a subject of interest because it leads to useful SAR conclusions and thus helps to synthesize pharmacologically active chalcones [1,2].

Recently, we prepared a large series of chalcones and showed that their potency against various yeast strains depended to a large extent on their ability to interact with yeast's intracellular thiols, such as glutathione and cysteine [3]. The studied chalcones had various functional groups in their ring B and belonged to three structural types in respect of their other ring A, namely chalcones with either no substitutions or possessing 4′-chloro or 3′,4′,5′-trimethoxy groups. We found that some of these compounds had been included in indexed sub-libraries subjected to antibacterial studies against several bacteria including *Staphylococcus aureus* and *Escherichia coli* [4]. The sub-libraries of chalcones with unsubstituted and methoxylated ring A showed one of the best inhibitory activities as was estimated by agar-well diffusion method. The same method was used for the determination of the minimal inhibitory concentration (MIC) of some of the individual chalcones. As such only one of the 3′,4′,5′-trimethoxychalcone series was synthesized and examined. To our knowledge, the examination of chalcones by only agar diffusion techniques may not be reliable enough, particularly for giving the quantitative measure of their antibacterial activity. The reason is that some of these compounds have a low rate of diffusion into the agar media [5]. This problem can be eliminated by using liquid media for the determination of the MICs of chalcones.

In this study, we enlarged the series of chalcones previously synthesized by us, preparing some novel hydroxylated compounds and chalcone derivatives with altered linker between the two aryl rings. Antibacterial effects of all the compounds

Abbreviations: ED, electron donating; EW, electron withdrawing.

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were investigated towards the most widely distributed pathogens in humans – *S. aureus* and *E. coli*. The MIC of the compounds was determined by the method of serial dilution in meat-peptone broth. Based on the results some structure–activity relationships were found and discussed in the light of existing hypotheses.

2. Chemistry

We prepared 62 chalcones and their derivatives by a Claisen–Schmidt condensation between acetophenones (acetophenone, *p*-chloroacetophenone and 3,4,5-trimethoxyacetophenone) or acetone and appropriate aryl aldehydes (Table 1). The desired products were obtained in yields between 50% and 95% after purification, following procedures described previously [3,6]. The structures were established with UV, IR, NMR, mass spectrometry and elemental analysis. ^{1}H NMR spectra showed that only *trans*-chalcones were obtained. Most of the chalcones were synthesized at 0 °C using sodium hydroxide as a catalyst. In this way we obtained 3-hydroxychalcone **9** with a yield of 92% in 24 h. Our attempt to shorten the reaction time by refluxing a reaction mixture of acetophenone and *m*-hydroxybenzaldehyde for 15 min resulted in only one product different from the expected chalcone. Its ^{1}H NMR spectrum showed no characteristic signals of the α,β-olefinic protons and 14 aromatic protons instead of the nine expected ones. In addition, two more signals were observed – two doublets of doublets for four protons at δ 3.40 and a pentet for one proton at δ 4.0. This information showed that at heating the reaction runs fast to receive the chalcone, which was further attacked by acetophenone to give the Michael adduct **56** (Fig. 1). Its structure was additionally confirmed by the ^{13}C NMR and mass spectral analyses. To our knowledge this compound has not been described in the literature up till now. The same applies to eight other compounds synthesized in this study (**33**, **44**, **50–54** and **61**). Their spectral data are presented in Section 5.

3. Microbiology

Antibacterial activity was checked by the agar-well diffusion method of Spooner and Sykes with *S. aureus* and *E. coli* grown on meat-peptone agar [7]. Chalcones and their derivatives giving an inhibitory zone with a diameter (d) of at least 15 mm were initially considered active. The minimal inhibitory concentration (MIC) of all compounds was determined by the method of serial dilutions as described by Hindler [8]. None of the compounds tested showed activity towards the Gram-negative bacterium *E. coli* while most of the chalcones inhibited the growth of the Gram-positive bacterium *S. aureus*. Four of the chalcones with anti-staphylococcal activity (compounds **30**, **40**, **44** and **45**) gave no inhibitory zones probably due to their low diffusion potential into the agar media. When tested in liquid media (meat-peptone broth) these compounds yielded MICs around 62.5 μg/ml.

4. Results, discussion and conclusion

Chalcones were not active towards *E. coli* in concentrations under 500 μg/ml. Interesting relationships were observed between the anti-staphylococcal activity of chalcones and their type of substitution. Presence of certain functional groups in the two aryl rings A and B was found to be of importance.

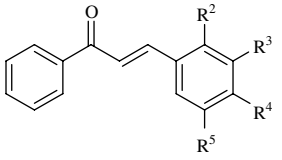
4.1. Electronic effects of the p-substituent in ring B

Mode of the antibacterial action of chalcones has long been believed to be due to their reaction with important thiol groups of essential enzymes via Michael addition at the ketovinyl double bond [9]. If this is the case, electronic effect of the *p*-substituent in ring B is of great importance. *p*-Electron withdrawing (EW) group is favourable because it will increase the electrophilicity of C-β and thus facilitate the nucleophilic attack of the cellular thiol groups. The opposite is true for the *p*-ED groups. To clarify if there is a relationship between the electronic effect of the *p*-substituent in ring B and the antibacterial activity of chalcones we studied 27 chalcones with *p*-EW or *p*-electron donating (ED) group as a single substituent in ring B. The results obtained for these compounds were compared to those obtained for chalcones with unsubstituted ring B. Also, three types of substitutions in ring A – X, Y and Z, were scrutinized. All the chalcones with *p*-ED groups in ring B were inactive under concentration of 500 μg/ml except for compounds **10** and **27** possessing *p*-OH group. Meanwhile, it was found that the presence of *p*-EW groups enhanced the activity only of chalcones with methoxylated ring A (Z-type) (Table 2).

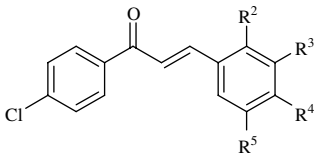
In order to understand the experimental results a detailed quantum chemical study was made on all 27 chalcones. Mulliken atomic charges of the C-β were calculated at B3LYP/6-31G* level of theory (Table 2). In most cases the presence of donor and acceptor groups in ring B had a direct influence on the charge of this C atom. However, this appeared not to be a determinant factor for the antibacterial activity of chalcones. The presence of three methoxy groups in ring A, which we found to be of great importance for the anti-staphylococcal activity, did not contribute to the changes in electrophilicity of C-β. In other words this electron parameter is not linked to the antibacterial activity of studied compounds.

We tried to correlate the MIC results of chalcones with other descriptors. Parameters such as refractivity, polarizability, mass, the coefficient of molecular partition octanol–water (log P), dipole moments, HOMO and LUMO energies, were disregarded due to their inability to differentiate the active compounds (data not given). A good correlation was observed with Δ, the energy difference between the two highest occupied molecular orbitals (HOMO and HOMO-1) (Table 2). This descriptor clearly discriminates between the active (MIC $\leq$ 250 μg/ml) and inactive (MIC = 500 μg/ml) chalcones (Fig. 2). The horizontal lines on the dendrogram (Fig. 2) represent the chalcones while the vertical ones show the similarity between pairs of them based on their MIC and Δ values. The parameter Δ has so far been successfully used for the prediction of diversified biological activities of

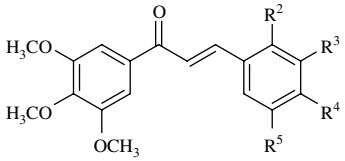
Table 1
Substitution pattern of the chalcones studied against *S. aureus*



X



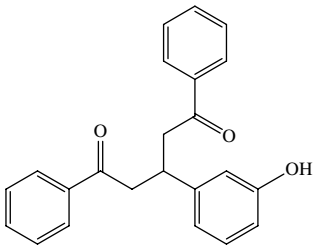
Y



Z

Chalcone	Type	R^2	R^3	R^4	R^5
1	X	H	H	H	H
2	X	H	H	Cl	H
3	X	H	$-OCH_2O-$		H
4	X	H	H	$N(CH_3)_2$	H
5	X	H	H	$NHCOCH_3$	H
6	X	H	H	NO_2	H
7	X	H	H	CN	H
8	X	OH	H	H	H
9	X	H	OH	H	H
10	X	H	H	OH	H
11	X	H	OH	OH	H
12	X	OH	OCH_3	H	H
13	X	H	OH	OCH_3	H
14	X	H	OCH_3	OH	H
15	X	H	OCH_3	H	H
16	X	H	H	OCH_3	H
17	X	H	H	CH_3	H
18	Y	H	H	H	H
19	Y	H	H	Cl	H
20	Y	H	$-OCH_2O-$		H
21	Y	H	H	$N(CH_3)_2$	H
22	Y	H	H	$NHCOCH_3$	H
23	Y	H	H	NO_2	H
24	Y	H	H	CN	H
25	Y	OH	H	H	H
26	Y	H	OH	H	H
27	Y	H	H	OH	H
28	Y	H	OH	OH	H
29	Y	OH	OCH_3	H	H
30	Y	H	OH	OCH_3	H
31	Y	H	OCH_3	OH	H
32	Y	H	OCH_3	OH	OCH_3
33	Y	OH	H	H	NO_2
34	Y	H	OCH_3	H	H
35	Y	H	H	OCH_3	H
36	Y	H	OCH_3	OCH_3	H
37	Y	H	OCH_3	OCH_3	OCH_3
38	Y	H	H	CH_3	H
39	Z	H	H	H	H
40	Z	H	H	Cl	H
41	Z	H	$-OCH_2O-$		H
42	Z	H	H	$N(CH_3)_2$	H
43	Z	H	H	$NHCOCH_3$	H
44	Z	H	H	NO_2	H
45	Z	H	H	CN	H
46	Z	H	OCH_3	H	H
47	Z	H	H	OCH_3	H
48	Z	H	OCH_3	OCH_3	H
49	Z	H	OCH_3	OCH_3	OCH_3
50	Z	OH	H	H	H
51	Z	H	OH	H	H
52	Z	H	H	OH	H
53	Z	H	OH	OCH_3	H
54	Z	H	OCH_3	OH	H
55	Z	H	H	CH_3	H

This table represents the structures of all the chalcones synthesized.



Compound **56**

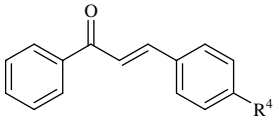
Fig. 1. The Michael adduct (compound **56**) obtained from the Claisen–Schmidt condensation between acetophenone and 3-hydroxybenzaldehyde under reflux.

thousands of compounds. It is supposed to be an indirect measure of the chemical reactivity and lifetime of excited states in the process of charge-transfer and/or covalent bond formation to cellular components [10].

4.2. Presence of hydroxyl group in ring B

Bowden et al. [11] supposed that chalcones inhibit the growth of *S. aureus* by interaction with an enzyme receptor consisting of two areas – a lipophilic region, associated with ring A and a hydrophilic region, which reacts with a hydroxyl group on ring B presumably by hydrogen bond formation. We studied 21 chalcones having hydroxylated ring B. All they possessed a lipophilic ring A, which was of structural type X, Y or Z (Table 3). Our results showed that the presence of hydroxyl group in ring B alone is not enough for the chalcones to exhibit anti-staphylococcal activity. Furthermore, the activity of chalcones was retained when an additional hydroxyl group was introduced (compounds **11** and **28**). However, the lipophilicity of ring A was found to be of importance. The hydroxyl chalcones of the less lipophilic Z-type were all inactive while the hydroxylated compounds of the more lipophilic X- and Y-types showed activity between 62.5 and

Table 2
EIM electronic variables



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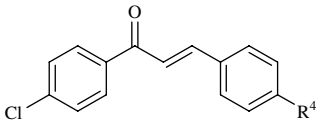

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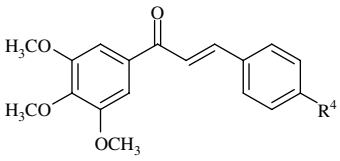


Z

Compound	Type	R^4	Charge on C-β	*H* (eV)	H-1 (eV)	Δ (eV)	d_{inh} (mm)	MIC (μg/ml)
1	X	H	−0.127	−8.424	−9.099	0.675	29 ± 1	125
6	X	NO_2	−0.138	−9.294	−9.396	0.102	17 ± 1	250
7	X	CN	−0.136	−8.991	−9.342	0.351	16 ± 0	500
2	X	Cl	−0.128	−8.559	−9.207	0.648	0	500
10	X	OH	−0.119	−8.019	−9.072	1.053	19 ± 1	125
17	X	CH_3	−0.123	−8.208	−9.072	0.864	0	500
16	X	OCH_3	−0.118	−7.943	−9.057	1.114	0	500
4	X	$N(CH_3)_2$	−0.114	−7.322	−8.978	1.656	0	500
5	X	$NHCOCH_3$	−0.125	−8.565	−8.982	0.417	0	500
18	Y	H	−0.124	−8.559	−9.207	0.648	0	500
23	Y	NO_2	−0.155	−7.074	−7.209	0.135	17 ± 1	250
24	Y	CN	−0.133	−9.126	−9.430	0.304	21 ± 1	250
19	Y	Cl	−0.125	−8.694	−9.585	0.891	0	500
27	Y	OH	−0.116	−8.127	−9.180	1.053	31 ± 1	125
38	Y	CH_3	−0.121	−8.316	−9.180	0.864	0	500
35	Y	OCH_3	−0.118	−8.058	−9.163	1.105	0	500
21	Y	$N(CH_3)_2$	−0.114	−7.416	−9.080	1.665	0	500
22	Y	$NHCOCH_3$	−0.125	−8.692	−9.051	0.359	0	500
39	Z	H	−0.128	−9.113	−9.261	0.148	0	250
44	Z	NO_2	−0.139	−8.586	−8.667	0.081	0	62.5
45	Z	CN	−0.138	−8.598	−8.679	0.081	0	62.5
40	Z	Cl	−0.128	−8.424	−8.505	0.081	0	62.5
52	Z	OH	−0.120	−8.046	−8.316	0.270	0	500
55	Z	CH_3	−0.124	−8.176	−8.494	0.318	0	500
47	Z	OCH_3	−0.120	−8.204	−8.519	0.314	0	500
42	Z	$N(CH_3)_2$	−0.121	−7.644	−8.409	0.765	0	500
43	Z	$NHCOCH_3$	−0.128	−8.131	−8.847	0.716	0	500

This table presents data for some EIM variables including energy difference HOMO – (HOMO-1), where HOMO refers to the highest occupied molecular orbital as well as data for the anti-staphylococcal activity of chalcones.
Mulliken atomic charges of the C-β; HOMO (*H*) and HOMO-1 (H-1) energies and contribution of their difference (Δ) for the antibacterial activity of chalcones of X, Y and Z-types having a single substituent at *p*-position in ring B.

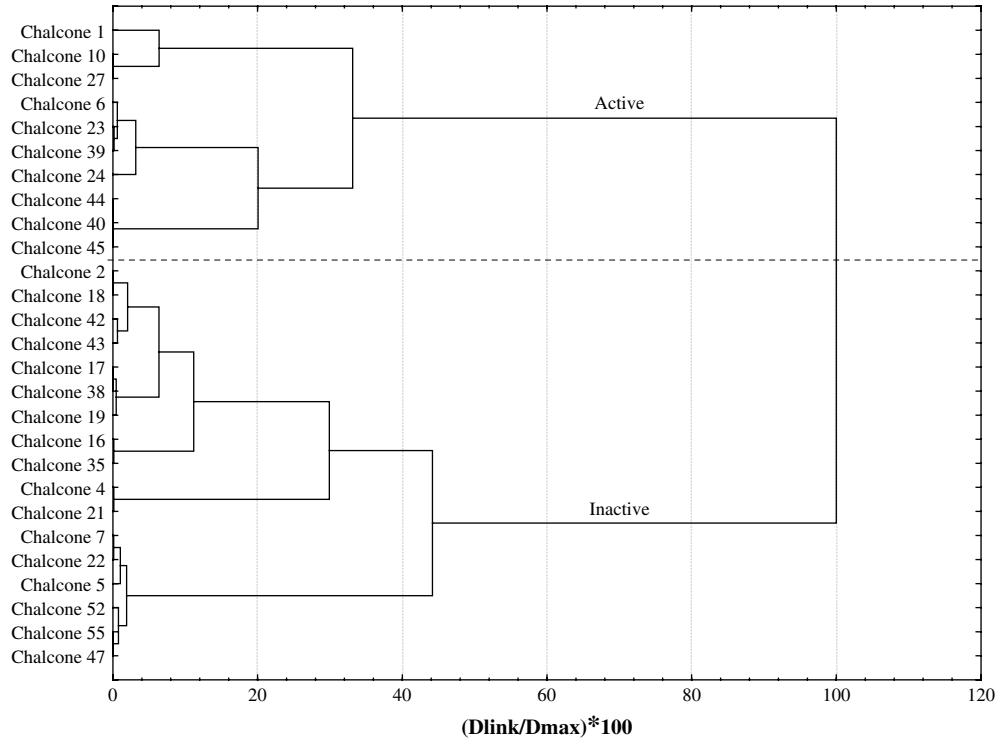
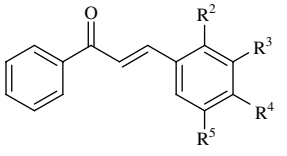
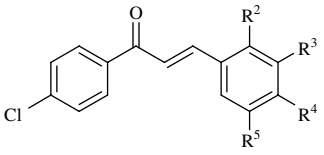


Fig. 2. Hierarchical dendrogram obtained with the Ward's method of linkage. It shows the distribution of active and inactive chalcones having one substituent at *p*-position in ring B, in respect of their Δ values.

Table 3
Effect of the position of the phenolic group and its surrounding in ring B on the chalcone activity against *S. aureus*



X



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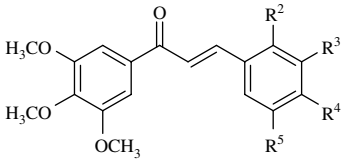


Z

Compound	Type	R^2	R^3	R^4	R^5	d_{inh} (mm)	MIC (μg/ml)
8	X	OH	H	H	H	22 ± 1	62.5
9	X	H	OH	H	H	20 ± 0	250
10	X	H	H	OH	H	19 ± 1	125
11	X	H	OH	OH	H	21 ± 1	125
12	X	OH	OCH_3	H	H	19 ± 1	125
13	X	H	OH	OCH_3	H	0	250
14	X	H	OCH_3	OH	H	0	500
25	Y	OH	H	H	H	20 ± 0	125
26	Y	H	OH	H	H	25 ± 0	125
27	Y	H	H	OH	H	31 ± 1	125
28	Y	H	OH	OH	H	19 ± 0	125
29	Y	OH	OCH_3	H	H	0	250
30	Y	H	OH	OCH_3	H	0	62.5
31	Y	H	OCH_3	OH	H	21 ± 1	250
32	Y	H	OCH_3	OH	OCH_3	19 ± 1	500
33	Y	OH	H	H	NO_2	0	500
50	Z	OH	H	H	H	0	500
51	Z	H	OH	H	H	0	500
52	Z	H	H	OH	H	0	500
53	Z	H	OH	OCH_3	H	0	500
54	Z	H	OCH_3	OH	H	0	500

This table presents data about the anti-staphylococcal activity of the chalcones having hydroxyl groups or a combination of hydroxyl and methoxyl groups in ring B.

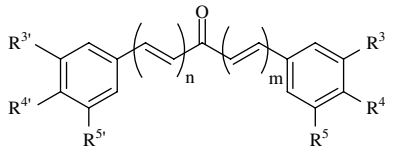
250 μg/ml. The position of hydroxyl group mattered only for the chalcones with unsubstituted ring A (X-type) as the *o*-position was most favourable, followed by $p = 3,4$ position and *m*-position. Introduction of a methoxy group next to *o*- and *p*-hydroxyl groups led to more inactive chalcones while the activity was kept when the methoxy group was inserted next to the *m*-hydroxyl group. Transformation of the *m*-hydroxychalcone to the Michael adduct (compound **56**) resulted in the loss of activity probably because in this way no Michael addition of thiol groups was possible.

Since previous papers highlighted the importance of the bulky substituents at C-5 in ring B for the antibacterial activity of chalcones we decided to introduce a nitro group at C-5 of the 4′-chloro-2-hydroxychalcone [12]. However, the synthesized chalcone **33** was not active.

4.3. Influence of the position of the keto-group and length of the conjugated linker between both aromatic rings

In some cases, structural alteration of chalcones, such as replacement of ring A or ring B by phenanthryl, naphthyl or pyridinium functions has resulted in a series of compounds with growth inhibitory properties to *S. aureus* and *E. coli* [1]. We decided to check if elongation of the conjugated linker between the aromatic rings of chalcones would affect their antibacterial activity. Thus, we synthesized six diphenyl alkenones (**57**–**62**) and examined their activity against *S. aureus*. These compounds can be regarded as chalcones in the molecules of which one or two double bonds were introduced either between ring A and the carbonyl function or between the carbonyl function and the double bond. It was observed that the insertion of one double bond between ring A and the carbonyl group led to compounds **60** and **61**, which were two-fold more active than the correspondent chalcones **19** and **49** (Table 4). The compounds with two double bonds from either one or both sides of the carbonyl group were inactive (MIC = 500 μg/ml).

Table 4
Effect of the change of the linker between the two aromatic rings on the activity against *S. aureus*



Entry	*n*	*m*	$R^{3\prime}$	$R^{4\prime}$	$R^{5\prime}$	R^3	R^4	R^5	d_{inh}[a]	MIC[a]
57	0	2	H	H	H	H	H	H	0	500
58	0	2	H	Cl	H	H	H	H	0	500
59	0	2	OCH_3	OCH_3	OCH_3	H	H	H	19 ± 1	500
60	1	1	H	Cl	H	H	Cl	H	0	250
61	1	1	OCH_3	OCH_3	OCH_3	OCH_3	OCH_3	OCH_3	0	250
62	2	2	H	H	H	H	H	H	0	500

This table presents data about the anti-staphylococcal activity of the chalcones with altered linker between their aromatic rings.

[a] The diameter of inhibitory zone d_{inh} is given in mm; the MIC unit is μg/ml.

In conclusion, the energy difference between the two highest occupied molecular orbitals (HOMO and HOMO-1) of chalcones with a single substituent at *p*-position in ring B differentiated the active compounds. Presence of hydroxyl group in ring B was not a determinant factor for the anti-staphylococcal activity, but the lipophilicity of ring A of the hydroxyl chalcones was of importance.

5. Experimental and computational details

Infrared and UV spectra were recorded on Bruker IFS 113 V and Helios gamma UV–vis spectrophotometers, respectively. ^1H and ^{13}C NMR spectra were obtained with Bruker AM 250 spectrometer with tetramethylsilane as internal reference. Chemical shifts are given in ppm (*d*-scale); coupling constants (J) are in Hz. Splitting patterns are described as singlet (s), doublet (d), triplet (t) and multiplet (m). Mass spectral analyses were accomplished on a Hewlett–Packard 5972 using Mass Selective Detector with EI (70 eV). The melting points were obtained using Mel-Temp 1102D-230 VAC and were uncorrected. The reactions were monitored on silica gel 60 F254 using petroleum ether/acetone 7:3 or toluene/Et_2O 4:1.

The quantum chemical calculations were performed using the GAUSSIAN-98 program package [13]. The geometries of all possible conformational isomers of studied chalcones were fully optimized at B3LYP/6-31G* level of theory without constraints. We used just the results for the most stable conformers according to the calculation in the analysis of structure–activity relationship.

5.1. Chemistry

5.1.1. General procedure for synthesis of chalcones

Acetophenone (97 μl, 0.8 mmol), *p*-chloroacetophenone (84 μl, 0.7 mmol) or 3,4,5-trimethoxyacetophenone (150 mg, 0.7 mmol) was added to equimolar quantities of appropriate aryl aldehydes or cinnamaldehyde and dissolved in MeOH (0.8 ml). To this solution 6 M NaOH (0.4 ml) was added and the reaction mixture was stirred for 40 min and then kept in refrigerator overnight. The product crystals were filtered and washed carefully with ice-water and cold MeOH to neutral reaction. The chalcones were purified by recrystallization.

*5.1.1.1. 4′-Chloro-2-hydroxy-5-nitrochalcone (**33**).* Yield 56%, orange crystals, m.p. 292–293 °C (MeOH). UV (MeOH): 287, 456 nm. IR (KBr): 3280 (OH), 1655 (C=O), 1595 (C=C), 836 (C–Cl) cm^{-1}. MS *m/z*: [$M^{+\bullet}$] 303 (100), $[M-17]^+$ 286 (65), $[M-35]^+$ 268 (20), $[M-111]^+$ 192 (57), $[M-164]^+$ 139 (60), $[M-139]^+$ 164 (45). ^1H NMR (DMSO-d_6, 250 MHz): δ 8.19 (d, $J = 15.3$ Hz, 1H, H-β), 8.02–8.07 (m, 2H, H-2′, H-6′), 7.94 (d, $J = 15.3$ Hz, 1H, H-α), 7.77 (dd, $J_1 = 8.3$ Hz, $J_2 = 2.0$ Hz, 1H, H-4), 7.64 (d, $J = 2.0$ Hz, 1H, H-6), 7.57–7.62 (m, 2H, H-3′, H-5′), 6.07 (t, $J = 7.4$ Hz, 1H, H-3). ^{13}C NMR (DMSO-d_6, 250 MHz): δ 189.0 (C=O), 174.4 (C-2), 144.2 (C-β), 138.9 (C-5), 137.5 (C-4), 137.2 (C-1′), 130.0 (C-2′, C-6′), 129.8 (C-α), 129.5

(C-3′, C-5′), 121.5 (C-6), 117.5 (C-1), 107.1 (C-3). Anal. for $C_{15}H_{10}ClNO_4$. Calcd (%): C, 59.32; H, 3.32; Cl, 11.67; N, 4.61. Found (%): C, 59.35; H, 3.37; Cl, 11.70; N, 4.65.

*5.1.1.2. 4-Nitro-3′,4′,5′-trimethoxychalcone (**44**).* Yield 83%, pale yellow crystals, m.p. 169–170 °C (MeOH). UV (MeOH): 278 nm. IR (KBr): 1650 (C=O), 1586 (C=C), 1325 (C–O–Ar) cm^{-1}. MS *m/z*: [$M^{+\bullet}$] 343 (100), [M − 15]$^+$ 328 (45), [M − 31]$^+$ 312 (20), [M − 43]$^+$ 300 (30), [M − 148]$^+$ 195 (32), [M − 167]$^+$ 176 (25), [M − 215]$^+$ 148 (28). ^{1}H NMR (DMSO-d_6, 250 MHz): δ 8.23–8.28 (m, 2H, H-3, H-5), 7.77 (d, $J = 15.8$ Hz, 1H, H-β), 7.63 (d, $J = 8.5$ Hz, 2H, H-2, H-6), 7.52 (d, $J = 15.8$ Hz, 1H, H-α), 7.18 (s, 2H, H-2′, H-6′), 3.93 (s, 3H, CH_3O), 3.91 (s, 6H, 2 × CH_3O). ^{13}C NMR (DMSO-d_6, 250 MHz): δ 189.3 (C=O), 154.3 (C-3′, C-5′), 148.1 (C-4), 145.6 (C-β), 145.2 (C-4′), 139.8 (C-1), 133.1 (C-1′), 128.2 (C-2, C-6), 122.4 (C-3, C-5), 121.0 (C-α), 107.8 (C-2′, C-6′), 56.4 (CH_3O), 56.0 (2 × CH_3O). Anal. for $C_{18}H_{17}NO_6$. Calcd (%): C, 62.97; H, 4.99; N, 4.08. Found (%): C, 63.01; H, 5.04; N, 4.15.

*5.1.1.3. 3-Hydroxy-3′,4′,5′-trimethoxychalcone (**51**).* Yield 82%, red crystals, m.p. 280–281 °C (MeOH). UV (MeOH): 315 nm. IR (KBr): 3425 (OH), 1639 (C=O), 1560 (C=C), 1375 (C–O–Ar) cm^{-1}. MS *m/z*: [$M^{+\bullet}$] 314 (37), [M − 15]$^+$ 299 (22), [M − 17]$^+$ 297 (86), [M − 31]$^+$ 283 (15), [M − 119]$^+$ 195 (100). ^{1}H NMR (DMSO-d_6, 250 MHz): δ 7.70 (d, $J = 15.8$ Hz, 1H, H-β), 7.59 (d, $J = 15.8$ Hz, 1H, H-α), 7.30 (t, $J = 8.2$ Hz, 1H, H-5), 7.20–7.26 (m, 3H, H-2′, H-6′, H-6), 7.10 (t, $J = 2.0$ Hz, 1H, H-2), 6.90 (dd, $J_1 = 2.0$ Hz, $J_2 = 8.2$ Hz, 1H, H-4), 3.95 (s, 3H, CH_3O), 3.93 (s, 6H, 2 × CH_3O). ^{13}C NMR (DMSO-d_6, 250 MHz): δ 189.3 (C=O), 159.0 (C-3), 153.1 (C-3′, C-5′), 145.6 (C-β), 143.2 (C-4′), 136.2 (C-1), 133.6(C-1′), 128.7 (C-5), 122.3 (C-α), 115.9 (C-4), 112.6 (C-2), 107.0 (C-2′, C-6′), 60.9 (CH_3O), 56.4 (2 × CH_3O). Anal. for $C_{18}H_{18}O_5$. Calcd (%): C, 68.78; H, 5.77. Found (%): C, 68.84; H, 5.82.

*5.1.1.4. 4-Hydroxy-3′,4′,5′-trimethoxychalcone (**52**).* Yield 62%, yellow crystals, m.p. 179–180 °C (MeOH). UV (MeOH): 350 nm. IR (KBr): 3430 (OH), 1650 (C=O), 1565 (C=C), 1380 (C–O–Ar) cm^{-1}. MS *m/z*: [$M^{+\bullet}$] 314 (29), [M − 15]$^+$ 299 (18), [M − 17]$^+$ 297 (75), [M − 31]$^+$ 283 (12), [M − 119]$^+$ 195 (100). ^{1}H NMR (DMSO-d_6, 250 MHz): δ 7.78 (d, $J = 15.5$ Hz, 1H, H-β), 7.68 (s, 4H, H-2, H-3, H-5, H-6), 7.40 (d, $J = 15.5$ Hz, 1H, H-α), 7.20 (s, 2H, H-2′, H-6′), 3.93 (s, 3H, CH_3O), 3.90 (s, 6H, 2 × CH_3O). ^{13}C NMR (DMSO-d_6, 250 MHz): δ 189.6 (C=O), 165.3 (C-4), 153.3 (C-3′, C-5′), 145.0 (C-β), 144.5 (C-4′), 133.4 (C-1′), 130.5 (C-1), 129.5 (C-2, C-6), 120.7 (C-α), 117.6 (C-3, C-5), 107.0 (C-2′, C-6′), 60.8 (CH_3O), 56.4 (2 × CH_3O). Anal. for $C_{18}H_{18}O_5$. Calcd (%): C, 68.78; H, 5.77. Found (%): C, 68.82; H, 5.84.

*5.1.1.5. 3-Hydroxy-3′,4,4′,5′-tetramethoxychalcone (**53**).* Yield 95%, brown crystals, m.p. 250–252 °C (MeOH). UV (MeOH): 260, 365 nm. IR (KBr): 3260 (OH), 1636 (C=O), 1561 (C=C), 1328 (C–O–Ar) cm^{-1}. MS *m/z*: [$M^{+\bullet}$] 344 (31), [M − 15]$^+$ 329 (18), [M − 31]$^+$ 313 (9), [M − 149]$^+$ 195 (100). ^{1}H NMR (CD_2Cl_2, 250 MHz): δ 7.69 (d, $J = 15.5$ Hz, 1H, H-β), 7.37 (d, $J = 15.5$ Hz, 1H, H-α), 7.20 (s, 2H, H-2′, H-6′), 7.15 (dd, $J = 8.1$, 2.0 Hz, 1H, H-6), 7.09 (d, $J = 2.0$ Hz, 1H, H-2), 6.77 (d, $J = 8.1$ Hz, 1H, H-5), 3.89 (s, 3H, CH_3O), 3.86 (s, 6H, 2 × CH_3O), 3.81 (s, 3H, CH_3O). ^{13}C NMR ($CDCl_3$, 250 MHz): δ 191.7 (C=O), 158.8 (C-4), 156.8 (C-3′, C-5′), 154.5 (C-4′), 149.4 (C-β, C-3), 135.6 (C-1, C-1′), 129.5 (C-α), 119.1 (C-6), 117.1 (C-5), 111.2 (C-2), 107.3 (C-2′, C-6′), 61.3 (CH_3O), 56.9 (2 × CH_3O), 55.8 (CH_3O). Anal. for $C_{19}H_{20}O_6$. Calcd (%): C, 66.27; H, 5.85. Found (%): C, 66.35; H, 5.92.

*5.1.1.6. 4-Hydroxy-3,3′,4′,5′-tetramethoxychalcone (**54**).* Yield 63%, white crystals, m.p. 75–76 °C (MeOH). UV (MeOH): 280 nm. IR (KBr): 3258 (OH), 1654 (C=O), 1585 (C=C), 1279 (C–O–Ar) cm^{-1}. MS *m/z*: [$M^{+\bullet}$] 344 (35), [M − 15]$^+$ 329 (20), [M − 31]$^+$ 313 (12), [M − 149]$^+$ 195 (100). ^{1}H NMR (CD_2Cl_2, 250 MHz): δ 7.66 (d, $J = 15.5$ Hz, 1H, H-β), 7.30 (d, $J = 15.5$ Hz, 1H, H-α), 7.19 (s, 2H, H-2′, H-6′), 7.17 (dd, $J_1 = 8.1$ Hz, $J_2 = 2.0$ Hz, 1H, H-6), 7.11 (d, $J = 2.0$ Hz, 1H, H-2), 6.87 (d, $J = 8.1$ Hz, 1H, H-5), 3.88 (s, 3H, CH_3O), 3.85 (s, 6H, 2 × CH_3O), 3.78 (s, 3H, CH_3O). ^{13}C NMR (CD_2Cl_2, 250 MHz): δ 189.1 (C=O), 153.6 (C-3′, C-5′, C-3), 148.7 (C-4′), 147.3 (C-4), 144.9 (C-β), 134.2 (C-1′), 127.9 (C-1), 123.4 (C-α), 119.8 (C-6), 115.0 (C-5), 110.6 (C-2), 106.3 (C-2′, C-6′), 60.9 (CH_3O), 56.7 (2 × CH_3O), 56.4 (CH_3O). Anal. for $C_{19}H_{20}O_6$. Calcd (%): C, 66.27; H, 5.85. Found (%): C, 66.52; H, 5.91.

*5.1.2. Preparation of 2-hydroxy-3′,4′,5′-trimethoxychalcone (**50**)*

Solid KOH (2.1 g, 37.5 mmol) was added to a mixture of 3,4,5-trimethoxyacetophenone (200 mg, 0.9 mmol) and salicyl aldehydes (101 μl, 0.9 mmol) in MeOH (2.5 ml) and H_2O (1.3 ml). The resulting solution was refluxed for 2 h, then cooled in an ice-water bath and acidified with 4.2 ml conc. HCl. The solution was diluted with distilled water (10 ml) and stored in a refrigerator overnight. The formed crystals were filtered, washed with water, dried out, purified by preparative thin-layer chromatography (petroleum ether:acetone 7:4) and recrystallized. Yield 50%, cream-colored crystals, m.p. 211–212 °C (MeOH). UV (MeOH): 280 nm. IR (KBr): 3490 (OH), 1650 (C=O), 1575 (C=C), 1375 (C–O–Ar) cm^{-1}. MS *m/z*: [$M^{+\bullet}$] 314 (34), [M − 15]$^+$ 299 (18), [M − 17]$^+$ 297 (80), [M − 31]$^+$ 283 (17), [M − 119]$^+$ 195 (100). ^{1}H NMR (DMSO-d_6, 250 MHz): δ 7.90 (d, $J = 15.8$ Hz, 1H, H-β), 7.63 (d, $J = 15.8$ Hz, 1H, H-α), 7.23–7.35 (m, 3H, H-4, H-5, H-6), 6.89 (s, 2H, H-2′, H-6′), 6.69 (d, $J = 8.0$ Hz, 1H, H-3), 3.96 (s, 3H, CH_3O), 3.93 (s, 6H, 2 × CH_3O). ^{13}C NMR (DMSO-d_6, 250 MHz): δ 189.6 (C=O), 158.9 (C-2), 153.4 (C-3′, C-5′), 145.0 (C-β), 144.1 (C-4′), 133.2 (C-1′), 130.0 (C-4), 128.7 (C-6), 122.5 (C-α), 120.7 (C-5), 116.9 (C-1), 107.3 (C-2′, C-6′), 60.9 (CH_3O),

56.4 (2 × CH_3O). Anal. for $C_{18}H_{18}O_5$. Calcd (%): C, 68.78; H, 5.77. Found (%): C, 68.83; H, 5.80.

*5.1.3. Preparation of 3-(3-hydroxyphenyl)-1,5-diphenylpentane-1,5-dione (**56**)*

m-Hydroxybenzaldehyde (98 mg, 0.8 mmol) and acetophenone (93 μl, 0.8 mmol) were dissolved in 0.8 ml MeOH and to this solution 0.4 ml of 6 M NaOH was added. The reaction mixture was then refluxed for 10 min. The product obtained was purified with column chromatography over silica using petroleum ether:acetone 7:1 to yield amorphous powder (**56**), yield 67%. UV (MeOH): 245, 280 nm. IR (KBr): 3375 (OH), 1657 (C═O) cm^{-1}. MS *m/z*: [$M^{+\bullet}$] 344 (7), $[M-18]^+$ 326 (8), $[M-119]^+$ 225 (100), $[M-239]^+$ 105 (95), $[M-267]^+$ 77 (59). ^1H NMR ($CDCl_3$, 250 MHz): δ 7.92–7.97 (m, 4H, Ar-H), 7.50–7.57 (m, 2H, Ar-H), 7.39–7.46 (m, 4H), 7.10 (t, $J = 8.01$ Hz, 1H, Ar-H), 6.78–6.81 (m, 2H, Ar-H), 6.63–6.67 (m, 1H, Ar-H), 6.14 (s, 1H, OH), 4.00 (p, $J = 7.01$ Hz, 1H, CH), 3.32, 3.47 (two dd, $J_1 = 16.5$ Hz, $J_2 = 7.0$ Hz, 4H, 2 × CH_2). ^{13}C NMR ($CDCl_3$, 250 MHz): δ 199.1 (2 × C═O), 156.1, 145.4, 136.7, 133.2, 129.8, 128.6, 128.3, 128.2, 128.0, 119.3, 114.6, 113.9, 44.8, 37.1, 30.9. Anal. for $C_{23}H_{20}O_3$. Calcd (%): C, 80.21; H, 5.85. Found (%): C, 80.02; H, 5.94.

*5.1.4. Synthesis of 1,5-bis-(3,4,5-trimethoxy-phenyl)-penta-1,4-dien-3-one (**61**)*

3,4,5-Trimethoxybenzaldehyde (294 mg, 1.5 mmol) was dissolved in 1.0 ml MeOH and acetone (53 μl, 0.73 mmol), and 1.4 ml of 6 M NaOH was added. In a few minutes solid formed. The product (**61**) was isolated after filtration, washing of the crystals with cold methanol and recrystallization. Yield 90%, green crystals, m.p. 108–110 °C (MeOH). UV (MeOH): 250, 365 nm. IR (KBr): 1610 (C═O), 1570 (C═C) cm^{-1}. MS *m/z*: [$M^{+\bullet}$] 414 (41), $[M-15]^+$ 399 (22), $[M-31]^+$ 383 (20), $[M-33]^+$ 181 (100). ^1H NMR ($CDCl_3$, 250 MHz): δ 7.66 (d, $J = 15.8$ Hz, 2H, 2 × H-β), 6.97 (d, $J = 15.8$ Hz, 2H, 2 × H-α), 6.84 (s, 4H, Ar-H), 3.910 (s, 12H, 4 × CH_3O), 3.89 (s, 6H, 2 × CH_3O). ^{13}C NMR ($CDCl_3$, 250 MHz): δ 188.5, 153.5, 143.3, 140.4, 130.2, 124.8, 105.6, 61.0 (2 × CH_3O), 56.2 (4 × CH_3O). Anal. for $C_{23}H_{26}O_7$. Calcd (%): C, 66.65; H, 6.32. Found (%): C, 66.85; H, 6.65.

5.2. Microbiology

The strains of *S. aureus* (Rosenbach 209) and *E. coli* (Castellani and Chalmers) WF^+ were obtained from the collection of the Stefan Angeloff Institute of Microbiology, Bulgarian Academy of Sciences and the Institute for State Control of Drugs, Sofia, Bulgaria.

5.2.1. Agar-well diffusion method

Two hundred-microliter suspension of the bacteria (10^5 cells/ml) was plated on the agar layer in Petri dishes (10 cm in diameter). Five wells per dish were prepared, each 10 mm in diameter. One hundred microliters of each sample, dissolved in 96% EtOH (5000 μg/ml) was added to appropriate wells. For pre-diffusion the Petri dishes were placed at 4 °C for 2 h. The antibacterial activity was estimated by the diameter of inhibitory zones formed in the agar layer after incubation at 37 °C for 48 h. Compounds giving an inhibitory zone with a diameter of at least 15 mm were considered active. Control experiments were carried out with the pure solvent.

5.2.2. Measurement of MIC

MIC was determined by serial dilution of each chalcone to 0.0–2000 μg/ml in test tubes using meat-peptone broth. Each test tube was inoculated with bacterial suspension containing 10^5 cells/ml and incubated at 37 °C for 24 h. The lowest dilution that visibly showed no growth compared to drug-free broth inoculated with microbial suspension was considered the MIC. For more precise detection, tubes that showed no visible growth were streaked on fresh meat-peptone agar plates, incubated at 37 °C for 24 h, and checked for growth.

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